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Effect of Fertilization on Expression of Bioactive Carotenoid and Chlorophyll Pigments in *Clinacanthus nutans* Lindau for Potential Use as Functional Natural Colourants

(Kesan Pembajaan terhadap Ekspresi Pigmen Karotenoid dan Klorofil Bioaktif pada *Clinacanthus nutans* Lindau untuk Potensi Kegunaan sebagai Pewarna Fungsian Semula Jadi)

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ABSTRACT

This research aimed to investigate the different effects of fertilization (through the use of vermicompost and chemical fertilizer) on carotenoid composition as well as the chlorophyll content in *Clinacanthus nutans* Lindau. A field study employing a Randomized Complete Block Design (RCBD) was conducted on four treatment groups; control plants that were not supplied with any fertilizer (NF), plants supplemented with NPK chemical fertilizer (FC), plants supplemented with vermicompost (FV), and plants supplied with the mixture of chemical fertilizer and vermicompost (FM). Vermicompost application was shown to have insignificant effects on the expression of chlorophyll and carotenoid in *C. nutans* as compared to chemical fertilizer. However, the supplementation of vermicompost (FV and FM) produced plants with greater stability of compounds during storage, in contrast to NF and FC. The major carotenoids in *C. nutans* methanolic extracts supplied with vermicompost and organic fertilizers were identified as violaxanthin, lutein, α-Carotene and β-Carotene, while control plants lacked in α-Carotene and β-Carotene. This analysis provides a better insight into the application of vermicompost as an alternative source of growth supplements for the sustainable production of pigments which can act as a functional natural colourant and deliver great medicinal benefits to humans.

Keywords: Carotenoid; *Clinacanthus nutans*; extract storage; total chlorophyll content; vermicompost

ABSTRAK

Penyelidikan ini bertujuan untuk mengkaji kesan berbeza pembajaan (melalui penggunaan vermikompos dan baja kimia) terhadap komposisi karotenoid serta kandungan klorofil dalam *Clinacanthus nutans* Lindau. Kajian lapangan yang menggunakan Reka Bentuk Blok Lengkap Rawak (RCBD) telah dijalankan ke atas empat kumpulan rawatan; tumbuhan kawalan yang tidak dibekalkan dengan sebarang baja (NF), tumbuhan yang ditambah dengan baja kimia NPK (FC), tumbuhan yang ditambah dengan vermikompos (FV) dan tumbuhan yang dibekalkan dengan campuran baja kimia dan vermikompos (FM). Penggunaan vermikompos telah ditunjukkan mempunyai kesan yang tidak ketara terhadap ekspresi klorofil dan karotenoid dalam *C. nutans* berbanding dengan baja kimia. Walau bagaimanapun, penambahan vermikompos (FV dan FM) menghasilkan tumbuhan dengan kestabilan sebatian yang lebih tinggi semasa penyimpanan, berbeza dengan NF dan FC. Karotenoid utama dalam ekstrak metanol *C. nutans* yang dibekalkan dengan vermikompos dan baja organik dikenal pasti sebagai violaxanthin, lutein, α-Karotena dan β-Karotena, manakala tumbuhan kawalan kekurangan α-Karotena dan β-Karotena. Analisis ini memberikan gambaran yang lebih baik tentang pengaplikasian vermikompos sebagai sumber alternatif tambahan tumbesaran untuk pengeluaran pigmen yang mampan yang boleh bertindak sebagai pewarna semula jadi yang berfungsi dan memberikan manfaat perubatan yang hebat kepada manusia.

Kata kunci: *Clinacanthus nutans*; jumlah kandungan klorofil; karotenoid; simpanan ekstrak; vermikompos

Introduction

Clinacanthus nutans Lindau or well-known as 'Belalai Gajah' among Malaysians is a type of medicinal plant from the Acanthaceae family that can be predominantly found in countries of Southeast Asia and also China. The plant has distinct characteristics which can be easily recognized based on its slightly curved stem, resembling the curve of an elephant's trunk, as well as its tall height which can grow up to 1 metre long. The leaves of *C. nutans* have potential medicinal uses in the treatment of several diseases such as kidney problems, skin irritation, herpes and herpes simplex virus (HSV) infection and lesions caused by the varicella-zoster virus (VSV) (Zainul Amiruddin et al. 2016). In addition to that, the leaves are also used as a relief for bites caused by snakes, insects and scorpions (Uawonggul et al. 2006). There have been numerous reports on the bioactivities of *C. nutans.* Besides possessing anti-inflammatory and antimicrobial activities, *C. nutans* also demonstrated anticancer, antivenom and strong antioxidant potential.

In recent years, researchers sought a greener and more sustainable approach for the production of agricultural as well as non-agricultural products. In agriculture, the use of the right fertilizer will not only ensure sufficient nutrients are made available to the plants and produce optimum plant yields, but will also aid in supplying the nutrients in the correct quantity and proportion, as well as in a usable form. The amount of essential plant pigments such as chlorophyll and carotenoid (Nasarudin et al. 2018) is also influenced by the type of fertilizers used, which are key for plant growth and development. Other than that, these pigments are also gaining interest for other applications such as in the development of functional natural colourants. Food colour is regarded as one of the key factors that influence consumer acceptance in the growing food and beverage industry (Giusti & Wallace 2009). Thus, plant-based pigments are gaining interest in the production of biomedical sensors, optical data storage, and photovoltaic (solar) cells as well as in the food industry as a replacement for synthetic dyes (Ayalew & Ayele 2016; El-Shishtawy 2009; Kim 2006; Mustroph, Stollenwerk & Bressau 2006).

In this study, vermicompost as an organic fertilizer was used to evaluate its effects on the accumulation of chlorophyll and carotenoid in *C. nutans.* Vermicompost is an organic fertilizer which contains low C-to-N ratios and is produced in a mesophilic process from the interaction between earthworms and microorganisms (Ramasamy & Suresh 2010). The application of vermicompost in the agricultural sector has been garnering a lot of interest due to its properties that contain a high amount of macro- and micronutrients, rich content of nitrogen, phosphorus and potassium as well as good soil microbes, thus making vermicompost a greener replacement for chemical fertilizers that are available in the market.

Previous studies on vermicompost had reported not only its ability to increase the resistance of plants towards pests and diseases, but also its positive effects on the growth and development of various plant species. Some of these species include medicinal plants (Chiluvuru et al. 2009), ornamentals (Chattopadhyay 2014; Sardoei et al. 2014), horticultural crops (Kashem et al. 2015; Sundararasu & Neelanarayanan 2012; Yang et al. 2013; Zucco et al. 2015), forestry species (Donald & Visser 1989; Lazcano et al. 2010b, 2010a) and also fruit crops (Acevedo & Pire 2004; Cabanas-Echevarría et al. 2005). These studies were focused more on plant growth as affected by the supplementation of vermicompost. However, to date, there is inadequate information available on the effects of vermicompost on the accumulation of chlorophyll and carotenoids in plants, especially on *C. nutans.* Thus, this study will help generate new knowledge and understanding of the availability of chlorophyll and carotenoid in *C. nutans* as a result of vermicompost supplementation. In addition to that, it will also aid in paving the way towards minimizing negative environmental effects while ensuring that these bioactive pigments are produced in a more sustainable approach.

Materials and Methods

Preparation of sample

The field study for this project was carried out at Universiti Malaya Centre of Biotechnology Research in Glami Lemi, Jelebu, Malaysia. There were four treatment groups involved and they consisted of control plants that were not supplied with any fertilizer (NF), plants supplemented with chemical fertilizer (NPK) at a concentration of 10 t ha^{-1}(FC), plants supplemented with organic fertilizer (vermicompost) at a concentration of 15 t ha^{-1} (FV), and plants supplemented with the mixture of chemical fertilizer and vermicompost (FM) at a concentration of 5 t ha^{-1} and 15 t ha^{-1}, respectively (Yusof et al. 2018). The vermicompost used in the study was readily prepared using a mixture of goat manure (GM) and spent mushroom compost (SMC) at GM:SMC of 80:20. The N, P and K concentrations in the vermicompost is as

follows; 1.29%-1.44% for Nitrogen (N), 0.72-0.74% for Phosphorus (P) and 1.5-2.07% for Potassium (K). *C. nutans* leaves were obtained on February 2017. The leaf samples were sent to the Rimba Ilmu herbarium at the Institute of Biological Sciences, Universiti Malaya, Kuala Lumpur, Malaysia for identification. Sample collections were dried for a week in an oven at 55 °C, mounted and identified by comparison with the voucher specimen (KLU 49509).

EXTRACTION OF CHLOROPHYLL AND CAROTENOID PIGMENTS

C. nutans leaves were freeze-dried at -50 °C with a freeze dryer (Labconco Corporation, MO 64132 United States) following the method by Yusof et al. (2018). Then, 3 g of the freeze-dried leaves were immersed in 90 mL of methanol and later pulverized using a chilled pestle and mortar. The solvent containing the sample mixture was added with a slight amount of $MgCO_3$ to avoid the turning of chlorophyll into phaeophytin under low pH. The sample was incubated for 24 h at 4 °C and then centrifuged for five minutes with a Universal 32 R centrifuge (Hettich Zentrifugen, D-78532 Germany) at 9050 × g at 4 °C. To prevent the degradation of pigments, the experiment was carried out under low light.

Then, to extract the carotenoid, 1.0 mL of distilled water was used to rehydrate 1.0 g of freeze-dried leaves of *C. nutans*. Five mL of solvents consisting of acetone and methanol at a ratio of 7 to 3 were used to soak the mixture overnight, without the presence of light at 22 °C. The mixture was vortexed before centrifugation at 13500 g for 2 min. Fifty mL graduated polypropylene centrifuge tubes were used to fill the collected supernatant and aluminium foil was used to cover the tubes, to minimize light exposure. After that, fine particulates in the supernatant were removed by centrifuging it for 5 min at 13500 g and kept at 4 °C, without the presence of light. An equal volume of distilled water and hexane was added and the solution was mixed with a vortex before centrifugation at 13500 g for a minute. Carotenoids that appeared in the top layer were collected and dried by passing them through a mild flow of oxygen-free nitrogen gas. Before storing the carotenoids at -80 °C, the vials containing the compounds were quickly covered and sealed with parafilm before analysis by HPLC.

SPECTROPHOTOMETRIC DETERMINATION OF TOTAL CHLOROPHYLL AND CAROTENOID CONTENT

Total chlorophyll (TCh) and carotenoid content (TC) were quantified following the previously described method by Lichenthaler and Buschmann (2005). The absorbance values at wavelengths 665.2, 652.4 and 470 nm were measured using a UV-200-RS spectrophotometer (MRC Ltd., Holon, Israel). The concentrations of chlorophyll a, chlorophyll b and total carotenoid were calculated based on the equation shown below as described by Lichtenthaler and Buschmann (2001):

$$\text{Chlorophyll } \alpha\ (\mu g/mL) = 16.72\ A_{665.2} - 9.16\ A_{652.4}$$

$$\text{Chlorophyll b } (\mu g/mL) = 34.09\ A_{652.4} - 15.28\ A_{665.2}$$

$$\text{Total carotenoid } (\mu g/mL) = (1000\ A_{470} - (1.63\ \text{Chl a}) - (104.96\ \text{Chl b}))/221$$

$$\text{Chlorophyll } a \text{ and chlorophyll } b \text{ weight ratio } \left(\frac{Ca}{Cb}\text{ratio}\right) = \frac{\text{Chlorophyll a}}{\text{Chlorophyll b}}$$

$$\text{Chlorophyll } a \text{ and } b \text{ to total carotenoid ratio} = \frac{C(a+b)}{C(x+c)}$$

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF CAROTENOIDS

The quantification of individual carotenoids in *C. nutans* extract was performed on an Agilent 1200 series HPLC system. Carotenoids were separated using a reverse phase ZORBAX SB-C_{18} column, 5 µm, 4.6 × 250 mm (Agilent Technologies, USA). This analysis utilized two types of mobile phases comprising the mixtures of (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The solvent gradients used were; 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. Re-equilibration of the column was done by using 100% solvent A for 10 min before the next injection was performed. 10 µL of injection volume was used and the temperature of the column was maintained at 20 ºC. Wavelengths ranging from 350 to 550 nm were used to detect the carotenoid peaks. The identity and amount of the carotenoids were determined based on the standard curves prepared using xanthophyll (neoxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, lycopene and lutein) and carotene (α-carotene and β-carotene) standards which were obtained from Sigma-Aldrich.

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The assessment of the antioxidant potential of *C. nutans* methanolic extracts was carried out using FRAP assay based on previously described methods, with minor

modifications (Benzie & Strain 1996; Kong et al. 2013). Briefly, FRAP reagent which consists of 300 mmol/L of acetate buffer (pH 3.6), 10 mmol/L 2,4,6,tripyridyl-s-triazine (TPTZ) in 40 mmol/L hydrochloric acid (HCl) and 20 mmol/L iron chloride ($FeCl_3$) were prepared. The TPTZ solution was mixed with acetate buffer, HCl and $FeCl_3$ at the ratio of 80:8:8:9.6 (v/ v/ v), respectively. Each well of the 96-well plate was filled with 10 µL of *C. nutans* extracts together with 300 µL of FRAP reagent. Subsequently, after 30 min of incubation, the absorbance values were recorded at 593 nm with a microplate reader (Multiskan Go, Thermo Scientific). The calibration curve was prepared using aqueous solutions of known ferrous ion concentration ($FeSO_4.7H_2O$) and the FRAP values were expressed as mmol Fe^{2+}/100 g sample. The antioxidant potential was assessed by the capacity of the extracts to reduce Ferric to Ferrous ions (Fe^{3+} to Fe^{2+}). The high reducing potential was denoted by the intense blue colour produced by the solution and it showed a linear relationship with the number of antioxidants present.

STABILITY OF PIGMENTS AFTER STORAGE OF EXTRACTS AT DIFFERENT TEMPERATURES

The effects of different fertilization as well as different storage conditions on the stability of extracts were analyzed in this study. *C. nutans* methanolic extracts from the four treatment groups (NF, FC, FV, FM) were stored for a period of 2 and 4 weeks at 2 different temperatures which were 4 °C and -20 °C. The temperatures were selected based on the common refrigerated/freezing storage temperatures. After storage, the chlorophyll and carotenoid contents together with their antioxidant activity were measured.

STATISTICAL ANALYSIS

Data obtained were analyzed by One-Way Analysis of Variance (ANOVA) while the differences between the pairs of means were measured using Duncan's Multiple Range Test (DMRT). The relationship between the bioactive compounds and the antioxidant potential of *C. nutans* extract was also determined using Pearson's correlation analysis.

RESULTS AND DISCUSSION

DETERMINATION OF PIGMENTS CONTENT (TOTAL CHLOROPHYLL AND TOTAL CAROTENOID CONTENT) AND ANTIOXIDANT POTENTIAL

The total chlorophyll (TCh) and total carotenoid (TC) contents in *C. nutans* methanolic extracts are shown in Table 1. Plants supplemented with chemical fertilizer (NPK) (FC) plants produced the highest total chlorophyll (6090.97 ± 144.90 µg/g dry weight) and carotenoid (520.47± 16.47 µg/g dry weight) contents among all the treatments. The chlorophyll *a* and chlorophyll *b* weight ratio (C*a*/C*b* ratio) as well as the chlorophyll *a* and *b* to total carotenoid (*C(a+b)/C(x+c)*) ratio were also calculated. Chl *a/b* ratios for NF, FC, FV and FM were 0.44, 0.45, 0.42 and 0.46, respectively (Table 2). Meanwhile, the *C(a+b)/C(x+c)* ratios for NF, FC, FV and FM were 12.43, 11.70, 16.62 and 17.28, respectively (Table 2).

TABLE 1. Total carotenoid and total chlorophyll contents in *C. nutans* methanolic extracts resulted from different plant growth supplements

Treatment	Sample ID	TC (µg/g DW)	TCh (µg/g DW)	Chl a content (µg/g DW)	Chl b content (µg/g DW)
No fertilizer	NF	470.19 ± 27.56^{b}	5845.19 ± 35.49^{ab}	1786.44 ± 60.85^{a}	4058.74 ± 29.75^{ab}
NPK fertilizer at 10 t ha^{-1}	FC	520.47± 16.47^{c}	6090.96 ± 144.90^{b}	1890.92 ± 94.10^{a}	4200.04 ± 92.65^{b}
Vermicompost at 15 t ha^{-1}	FV	359.40 ± 11.63^{a}	5975.00 ± 38.23^{ab}	1761.24 ± 72.93^{a}	4213.76 ± 54.72^{b}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	FM	335.70 ± 6.82^{a}	5801.34 ± 70.29^{a}	1824.34 ± 71.10^{a}	3977.00 ± 6.36^{a}

DMRT test shows that means on the same column denoted by different alphabets differ significantly at $p \leq 0.05$

The identification of individual carotenoids in the plant extracts was carried out by HPLC analysis. There were four types of carotenoids detected and they include xanthophylls comprising violaxanthin and lutein, as well as carotenes consisting of α-Carotene and β-Carotene (Figure 1). Interestingly, the addition of growth substrates was found to boost carotenoid content and composition in *C. nutans*. Plants grown

TABLE 2. The ratios of chlorophyll a to chlorophyll b (C*a*/C*b*) and total chlorophyll to total carotenoid (*Ca + Cb)/(C (x + c)*) ratios of *C. nutans* methanolic extracts resulted from different plant growth supplements

Treatment	Sample ID	Ratio of Ca/Cb (µg/g DW)	Ratio of (Ca + Cb)/ (C (x + c)) (µg/g DW)
No fertilizer	NF	0.44 ± 0.02[a]	12.82 ± 0.84[a]
NPK fertilizer at 10 t ha^{-1}	FC	0.45 ± 0.02[a]	11.84 ± 0.57[a]
Vermicompost at 15 t ha^{-1}	FV	0.42 ± 0.02[a]	16.77 ± 0.57[b]
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	FM	0.46 ± 0.02[a]	17.34 ± 0.43[b]

DMRT test shows that means on the same column denoted by different alphabets differ significantly at p≤0.05

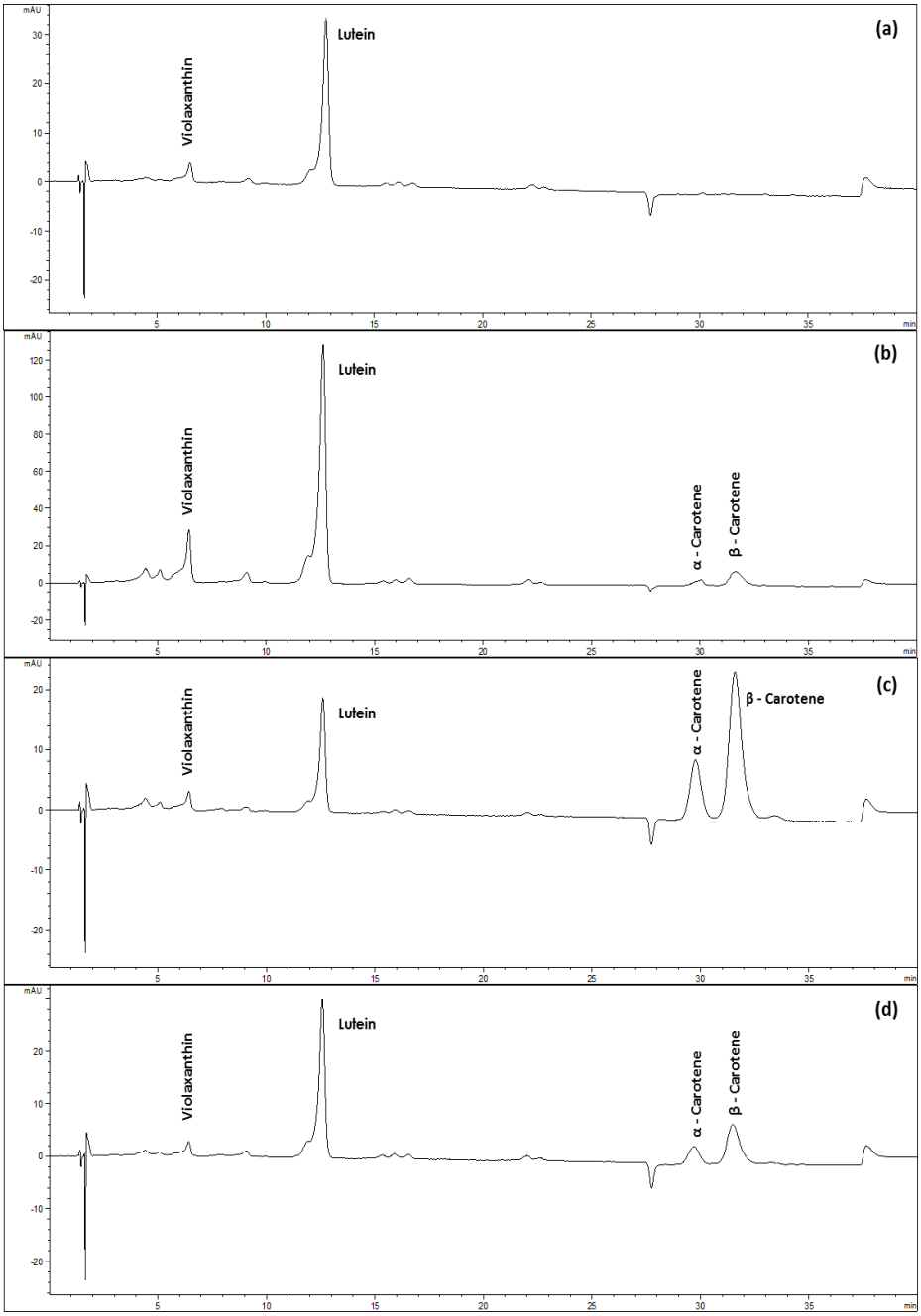

FIGURE 1. HPLC chromatograms of (a) NF, (b) FC, (c) FV and (d) FM plant extracts showing the presence of violaxanthin, lutein, α-carotenoid and β-carotenoid

without fertilizer (control; NF) were found to be lacking in α-Carotene and β-Carotene. Meanwhile, the application of vermicompost resulted in a higher amount of α-Carotene and β-Carotene as compared to plants with no fertilizer and plants supplemented with chemical fertilizer (Table 3). Contrarily, the highest amount of violaxanthin (5.73 ± 0.22 µg/g dry weight) and lutein (551.70 ± 4.13 µg/g dry weight) was observed in FC plants (Table 3). Englert et al. (1995) reported that the pigments contained within green leafy vegetables are constituted by the major carotenoids such as lutein and violaxanthin, similar to the carotenoids present within the leaves of *C. nutans.*

TABLE 3. Composition and content of carotenoid in *C. nutans* methanolic extract by HPLC analysis

Treatment	Sample ID	Composition and content of carotenoid in *C. nutans* methanolic extracts (µg/g DW)			
		Violaxanthin	Lutein	α- Carotene	β-Carotene
No fertilizer	NF	4.33 ± 0.56^{ab}	209.42 ± 20.95^{a}	ND	ND
NPK fertilizer at 10 t ha^{-1}	FC	5.73 ± 0.22^{b}	551.70 ± 4.13^{b}	11.83 ± 1.11^{b}	9.43 ± 0.91^{b}
Vermicompost at 15 t ha^{-1}	FV	4.37 ± 0.62^{ab}	206.78 ± 40.31^{a}	25.70 ± 4.31^{c}	20.67 ± 3.96^{c}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	FM	3.75 ± 0.03^{a}	189.52 ± 22.03^{a}	24.40 ± 4.26^{c}	14.77 ± 1.98^{bc}

DMRT test shows that means on the same column denoted by different alphabets differ significantly at $p \leq 0.05$.
*ND not detected

FRAP (ferric reducing antioxidant power) assay was carried out to determine the antioxidant potential of *C. nutans* extracts and the results were expressed as mg per mL $FeSO_4$ equivalent of each extract. Based on data analysis, the highest reducing potential was observed in the methanolic extract from control plants (NF) with FRAP values of 12.25 ± 0.66 mg/mL followed by FC, FV and FM (Table 4). The FRAP values of FC, FV and FM differ significantly from NF at $p \leq 0.05$.

TABLE 4. Antioxidant potential of *Clinacanthus nutans* methanolic extracts as results of different plant growth supplements

Treatment	Sample ID	FRAP values (mg/mL)
No fertilizer	NF	12.16 ± 0.18^{b}
NPK fertilizer at 10 t ha^{-1}	FC	10.35 ± 0.65^{a}
Vermicompost at 15 t ha^{-1}	FV	10.17 ± 0.01^{a}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	FM	10.10 ± 0.07^{a}

DMRT test shows that means on the same column denoted by different alphabets differ significantly at $p \leq 0.05$

STABILITY OF PIGMENTS AND ANTIOXIDANT PROPERTIES AFTER EXTRACT STORAGE AT DIFFERENT TEMPERATURES AND DURATION

In this study, the effects of storage at different temperatures after 2 and 4 weeks on *C. nutans* methanolic extracts were studied. As observed in Figure 2, the results showed that chlorophyll (C*a*) content in FV exhibited the least reduction, with a decrease of 10.29% when stored for 2 weeks at -20 °C and 22.41% at 4 °C. After 4 weeks of storage, C*a* for FV extract further decreased by 45.70% and 53.84% when stored at -20 °C and 4 °C, respectively. *C. nutans* extracts contained higher amounts of Chlorophyll b (C*b*) than C*a* as shown in the results. FV plant extracts exhibited the least reduction in the level of C*b* with 25.94% and 60.76% losses during storage at 4 °C for 2 and 4 weeks, compared to NF plants (63.89% and 68.23% losses, respectively). After 4 weeks of storage, FV showed a reduction in C*b* content by more than 60% during storage at both storage temperatures.

FV plant extract was found to be more stable when kept at different storage conditions, as evident by the lower reduction of total chlorophyll levels compared to extracts from other treatments. TCh for FV extracts were found to decline by 21.37% and 24.90% when stored for 2 weeks at -20 °C and 4 °C, respectively. In comparison, FC exhibited the highest decline in the chlorophyll levels with over 60% decrease in TCh after 4 weeks of extract storage at 4 °C. Chemical fertilizers

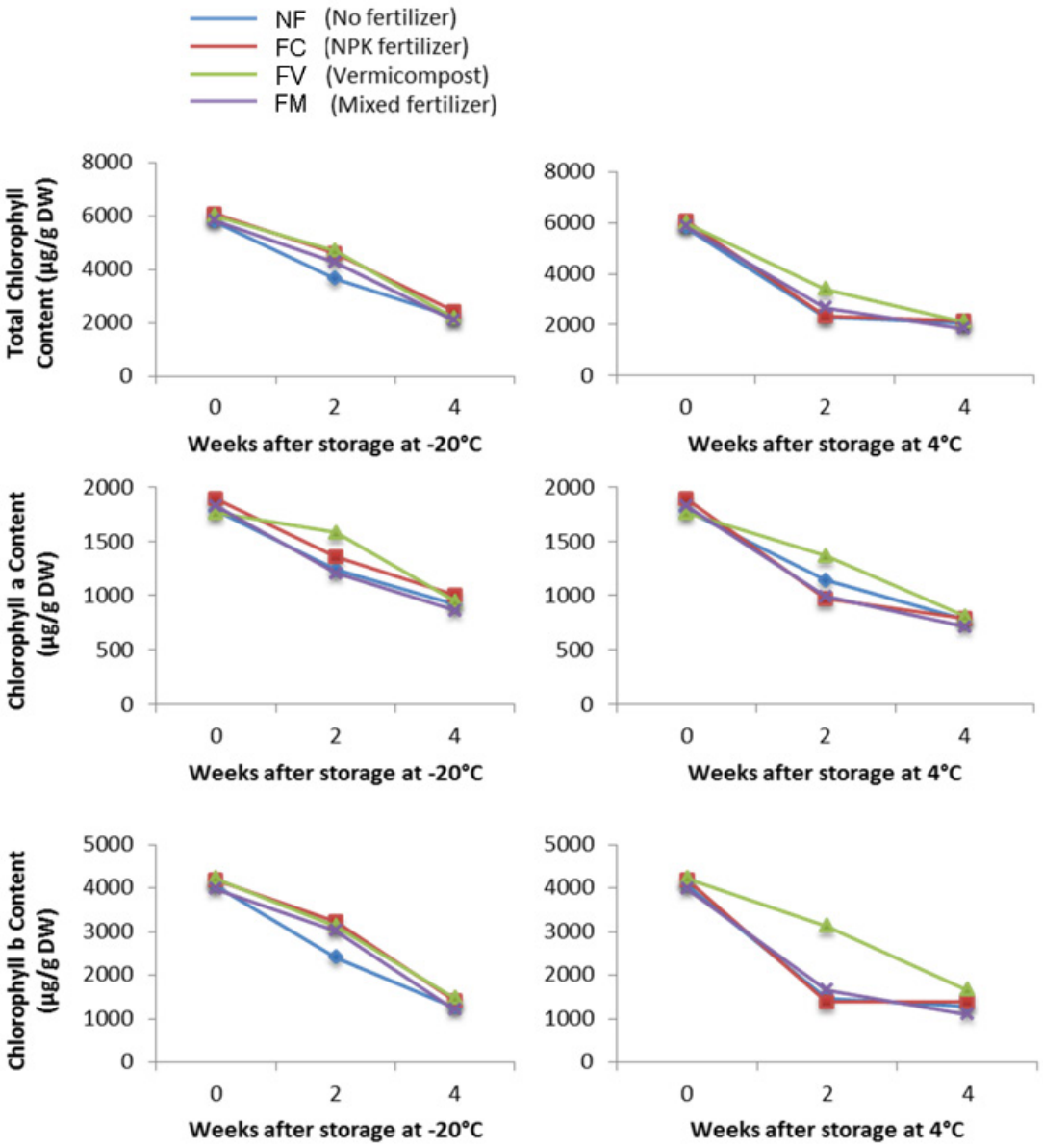

FIGURE 2. Total chlorophyll (TCh), chlorophyll a and chlorophyll b contents in *C. nutans* methanolic extracts supplemented with different growth supplements during storage at -20 °C and 4 °C

are an important input that can help increase agricultural productivity; nevertheless, using a high dose of chemical fertilizers has been linked to a decline in both soil quality and crop yields over the long term. On the other hand, organic fertilizers like cow manure and compost have been shown to have a positive impact on the characteristics of the soil (Purbajanti, Slamet & Fuskhah 2019). Hence, this may explain why plants supplemented with chemical fertilizer in this present study showed a higher decline in chlorophyll over time as compared to vermicompost-treated plants.

Ferrante and Maggiore (2007) in their study on leafy vegetables found that there was a reduction in total chlorophyll content with 22% of losses when stored at 4°C after 8 days. Another study carried out by Raya et al. (2015) found that there was a reduction of total chlorophyll content in different parts of *C. nutans* with 35, 62 and 78% of losses after 2, 3 and 4 days of storage, respectively, when they are kept at much higher temperature 25 °C. The reduction in the total content of chlorophyll is affiliated with several surrounding factors, for example, high temperature, exposure to light, moisture, senescence, ripening as well as other conditions such as the breakdown of chloroplast and degradation of chlorophyll (Meir et al. 1992; Roura, Davidovich & Valle 2000). Changes in the content of chlorophyll also result from the effects of physicochemical changes that occur during post-crops harvesting and storage (Wrolstad et al. 1995). The degradation of chlorophyll is reported to be initiated by an enzyme named chlorophyllase, which caused senescence in plant leaves (Matile, Schellenberg & Vicentini 1997). Results from this study also showed that all plant extracts contained a higher concentration of chlorophyll *b* as compared to chlorophyll *a*, which is in agreement with previous studies conducted by Ali et al. (2018) and Ling et al. (2009). Chlorophyll *b* can be exclusively found in the complexes of the antenna system while chlorophyll *a* is located in the photosystems reaction centres in the pigment antenna (Lichtenthaler & Buschmann 2001).

As depicted in Figure 3, the total carotenoid content increased during storage at different temperatures. After 4 weeks of storage at -20 °C and 4 °C, FV extracts showed a higher increase of TC as compared to NF and FC. NF on the other hand showed the lowest increment in TC with only 47.10% and 36.98% when extracts were stored at -20 °C and 4 °C, respectively, for 4 weeks. Noseworthy and Loy (2008) obtained similar findings in which the total carotenoid content for *Cucurbita maxima* squash increased after the extract was stored at 15 °C for 30 days.

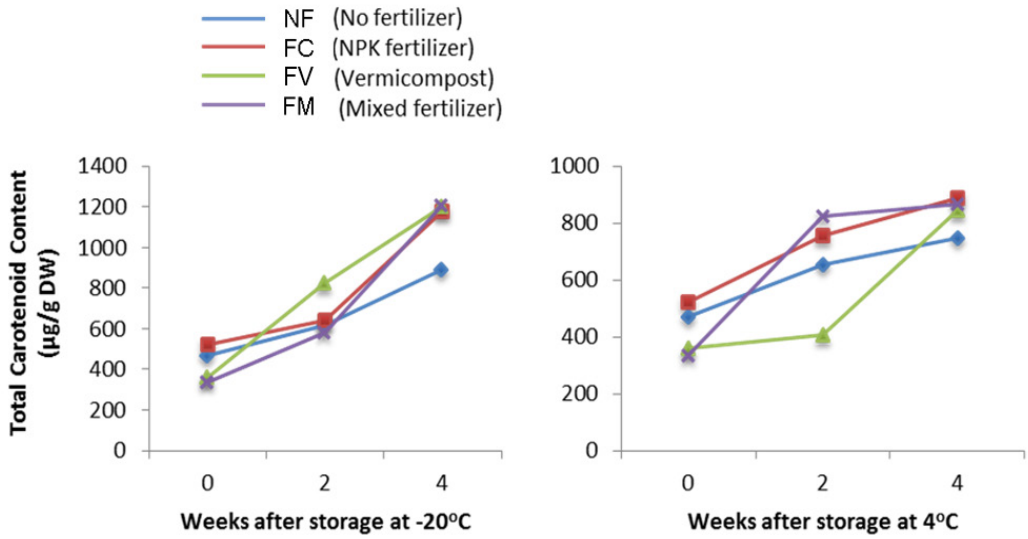

FIGURE 3. Total carotenoid content (TC) in *C. nutans* methanolic extracts supplemented with different growth supplements during storage at different temperatures

Results from a study by Ali et al. (2018) demonstrated an increase in total carotenoid content in the coloured calli of *Orthosiphon stamineus* extracts when stored at -20°C for 4 weeks. The author suggested that compared to chlorophylls, carotenoids were less susceptible to degradation at low temperatures (Ali et al. 2018). The

increase in carotenoids during storage can also be referred to as post-maturation or after-ripening (Noseworthy & Loy 2008). Results from this study indicate that the carotenoid synthesis in *C. nutans* continues to function long after harvest, and the system is enhanced by storage at low temperatures.

Table 5 shows the ratio of pigment contents in *C. nutans* methanolic extracts after extract storage at -20 °C and 4 °C. After 4 weeks of storage at -20 °C, *C(a+b)/C(x+c)* ratio decreased to 2.46, 2.05, 2.03 and 1.71 for NF, FC, FV, and FC, respectively. The ratio of *C(a+b)/C(x+c)* reduced to 2.77, 2.45, 2.92, and 2.11 for NF, FC, FV and FM, respectively, after the extracts were stored at 4 °C for 4 weeks. The constant decline of the *C(a+b)/C(x+c)* ratio may be due to the chromoplast development which is indicated by the yellowing colour of the plant leaves (Giusti & Wrolstad 2001). Besides that, lower values of the *C(a+b)/C(x+c)* ratio indicate a higher degradation rate of chlorophylls compared to carotenoids which are associated with stress, plant ageing and impairment of the protein-pigment complexes. As senescence progressed, the leaves displayed values of 3.5 or even lower for the *C(a+b)/C(x+c)* ratio (Lichtenthaler & Buschmann 2001). The high concentration of chlorophyll *b* obtained in this study also resulted in a lower ratio of chlorophyll *a* to chlorophyll *b* (*Ca/Cb*). It was reported that exposure of leaves to direct sunlight resulted in a lower value of *Ca/Cb* ratio because high irradiance causes faster degradation of chlorophyll *a* as compared to chlorophyll *b*. This happened due to the photosynthetic mechanism which offers little protection to chlorophyll *a* (Morais et al. 2007). Goodwin (1980) and Wolf (1956) also agreed that chlorophyll *a* deteriorate faster than chlorophyll *b*. Due to this fact, sun-exposed leaves showed lower chlorophyll levels in comparison with shade leaves (Boardman 1977). Furthermore, the increase in the size of the pigment antenna system present in photosystem II could also result in the decreased value of *the Ca/Cb* ratio (Lichtenthaler & Buschmann 2001). However, a contrary result was reported by Guzman, Yousef and Brown (2012) who obtained a 4 times higher level of chlorophyll *a* to chlorophyll *b*.

TABLE 5. Ratio of pigment contents in methanolic extracts of *C. nutans* Lindau before and after extract storage at various temperatures

Treatment	Sample ID	C*a*/C*b* ratio (mg/g DW)		(C*a* + C*b*)/ (C (x + c)) ratio (mg/g DW)	
		-20 °C	4 °C	-20 °C	4 °C
No fertilizer	NF_0	0.44	0.44	12.30	12.30
	NF_{W2}	0.52	0.78	5.85	4.00
	NF_{W4}	0.72	0.60	2.46	2.77
NPK fertilizer at 10 t ha^{-1}	FC_0	0.45	0.45	11.70	11.70
	FC_{W2}	0.42	0.70	7.18	3.12
	FC_{W4}	0.71	0.57	2.05	2.45
Vermicompost at 15 t ha^{-1}	FV_0	0.42	0.42	16.62	16.62
	FV_{W2}	0.51	0.44	5.71	11.04
	FV_{W4}	0.71	0.49	2.03	2.92
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	FM_{w0}	0.46	0.46	17.36	17.36
	FM_{W2}	0.40	0.60	7.30	3.22
	FM_{W4}	0.73	0.65	1.71	2.11

The indicators (0, W2, W4) written as subscripts following sample ID indicate the storage conditions (0 – initial; W2 – after 2-week storage; W4 – after 4-week storage).
*C*a* chlorophyll *a*, C*b* chlorophyll *b*, C*a* + C*b* total chlorophyll *a* and *b*, C(x+c) total carotenoid (xanthophyll and carotene)

The effects of different storage conditions on *C. nutans* antioxidant potential are depicted in Figure 4. It was found that storage temperature and duration significantly influenced the reducing potential of *C. nutans* methanolic extract. The reducing potential decreased by 16.20%, 19.03%, 14.85%, and 16.14%, respectively, for NF, FC, FV, and FM after the extracts were stored for 2 weeks at -20 °C. The reducing potential decreased further during storage for 4 weeks at -20 °C, with FV extracts exhibiting the least reduction (41.99%) compared to other extracts. These results indicate that FV extracts were more stable when subjected to prolonged storage. Moreover, the reducing potential of all extracts also decreased significantly after extract storage at 4 °C. The reducing potential of all extracts decreased by more than 50% with FV showing 54.08% losses and NF, FC and FM exhibiting 58.88%, 65.51% and 60.20% of losses, respectively, after 2 and 4 weeks of storage.

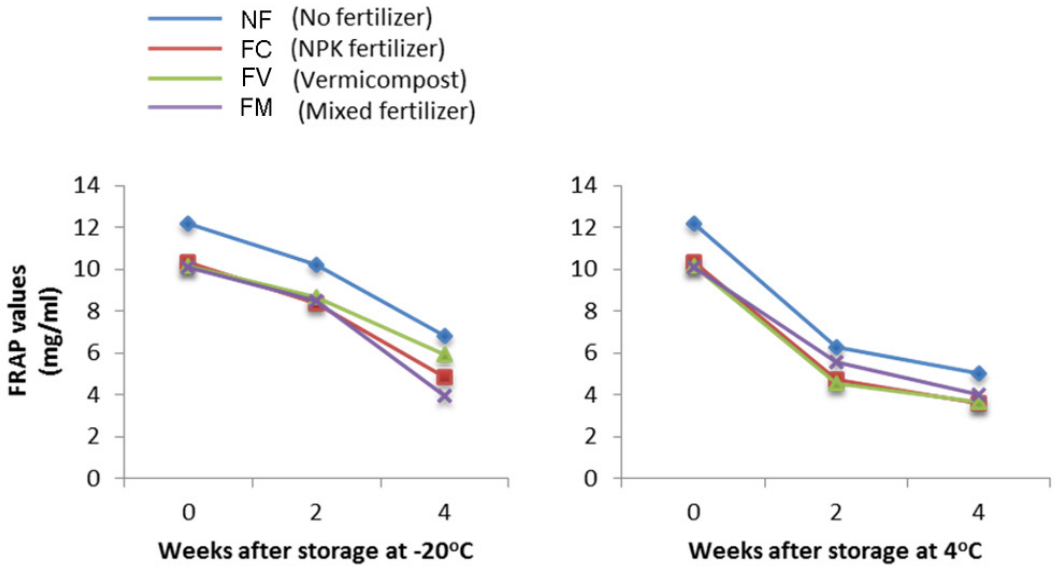

FIGURE 4. Antioxidant activities of *C. nutans* methanolic extracts supplemented with different growth supplements during storage at -20 °C and 4 °C

CORRELATION ANALYSIS

The correlation between total chlorophyll (TCh) and total carotenoid (TC) contents of *C. nutans* methanolic extracts from different treatment groups and their antioxidant potential were studied using Pearson correlation analyses. TCh showed a significant correlation with FRAP values (r = 0.834), indicating that higher chlorophyll contents resulted in increasing reducing potential (Table 6). However, TC exhibited a significant negative correlation with FRAP values (r = -0.553). This was possibly due to the continuous increase of carotenoid levels in *C. nutans* extracts after storage, although the antioxidant activity was decreased.

TABLE 6. Correlation between bioactive compounds composition with antioxidant properties of *C. nutans* methanolic extract

	TCh	TC	FRAP
TCh	1		
TC	-0.685**	1	
FRAP	0.834**	-0.553**	1

** Correlation is significant at p<0.01, * Correlation is significant at p<0.05, *TCh* total chlorophyll content; *TC* total carotenoid content; *FRAP* ferric reducing antioxidant power

CONCLUSIONS

The contents of chlorophyll and carotenoid of *C. nutans* leaves were not significantly influenced by the supplementation of vermicompost. However, this study showed that the number of compounds produced by *C. nutans* supplied with chemical fertilizer was as high as the control. Different storage conditions significantly affect the stability of compounds, with plants supplemented with vermicompost showing better stability during storage as compared to control plants and plants supplemented with chemical fertilizer.

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REFERENCES

Acevedo, I.C. & Pire, R. 2004. Effects of vermicompost as substrate amendment on the growth of papaya (*Carica papaya* L.). *Interciencia* 29(5): 274-279.

Ali, H., Karsani, S.A., Othman, R. & Yaacob, J.S. 2018. Production of coloured callus in *Orthosiphon stamineus* Benth and antioxidant properties of the extracted pigments. *Pigment and Resin Technology* 47(3): 196-207.

Ayalew, W.A. & Ayele, D.W. 2016. Dye-sensitized solar cells using natural dye as light-harvesting materials extracted from *Acanthus sennii* chiovenda flower and *Euphorbia cotinifolia* leaf. *Journal of Science: Advanced Materials and Devices* 1(4): 488-494.

Benzie, I.F.F. & Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry* 239(1): 70-76.

Boardman, N.K. 1977. Comparative photosynthesis of sun and shade plants. *Annual Review of Plant Physiology* 28: 355-377.

Cabanas-Echevarría, M., Torres-García, A., Díaz-Rodríguez, B., Ardisana, E.F.H. & Creme-Ramos, Y. 2005. Influence of three bioproducts of organic origin on the production of two banana clones (*Musa* spp AAB.) obtained by tissue cultures. *Alimentaria* 369: 111-116.

Chattopadhyay, A. 2014. Effect of vermiwash and vermicompost on an ornamental flower, *Zinnia* sp. *Journal of Horticulture* 1: 112.

Chiluvuru, N., Tartte, V., Kalla, C.M. & Kommalapati, R. 2009. Plant bioassay for assessing the effects of vermicompost on growth and yield of *Vigna radiata* and *Centella asiatica*, two important medicinal plants. *Journal of Developments in Sustainable Agriculture* 4(2): 160-164.

Donald, D.G.M. & Visser, L.B. 1989. Vermicompost as a possible growth medium for the production of commercial forest nursery stock. *Applied Plant Science* 3(2): 110-113.

El-Shishtawy, R.M. 2009. Functional dyes, and some hi-tech applications. *International Journal of Photoenergy* 2009: 434897. doi: 10.1155/2009/434897.

Englert, G., Aakemann, T., Schiedt, K. & Liaaen-Jensen, S. 1995. Structure elucidation of the algal carotenoid (3S, 5R, 6R, 3‘S, 5‘R, 6‘S)-13‘-cis-7‘, 8‘-dihydroneoxanthin-20‘-al 3‘-β-lactoside (P457). Part 2, NMR studies. *Journal of Natural Products* 58(11): 1675-1682.

Ferrante, A. & Maggiore, T. 2007. Chlorophyll a fluorescence measurements to evaluate storage time and temperature of Valeriana leafy vegetables. *Postharvest Biology and Technology* 45(1): 73-80.

Giusti, M.M. & Wrolstad, R.E. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*. F1.2.1 - F1.2.13. New York: John Wiley & Sons, Inc.

Giusti, M.M. & Wallace, T.C. 2009. Flavonoids as natural pigments. In *Handbook of Natural Colorants*. New York: John Wiley & Sons, Ltd. pp. 255-275.

Goodwin, T.W. 1980. Biogeochemistry of carotenoids. In *The Biochemistry of the Carotenoids*. Dordrecht: Springer. pp. 346-349.

Guzman, I., Yousef, G.G. & Brown, A.F. 2012. Simultaneous extraction and quantitation of carotenoids, chlorophylls, and tocopherols in Brassica vegetables. *Journal of Agricultural and Food Chemistry* 60(29): 7238-7244.

Kashem, M.A., Sarker, A., Hossain, I. & Islam, M.S. 2015. Comparison of the effect of vermicompost and inorganic fertilizers on vegetative growth and fruit production of tomato (*Solanum lycopersicum* L.). *Open Journal of Soil Science* 5(2): 53.

Kim, S.H. 2006. *Functional Dyes*. Elsevier Science.

Kong, K.W., Khoo, H.E., Prasad, N.K., Chew, L.Y. & Amin, I. 2013. Total phenolics and antioxidant activities of *Pouteria campechiana* fruit parts. *Sains Malaysiana* 42(2): 123-127.

Lazcano, C., Sampedro, L., Zas, R. & Domínguez, J. 2010a. Assessment of plant growth promotion by vermicompost in different progenies of maritime pine (*Pinus pinaster* Ait.). *Compost Science & Utilization* 18(2): 111-118.

Lazcano, C., Sampedro, L., Zas, R. & Domínguez, J. 2010b. Vermicompost enhances germination of the maritime pine (*Pinus pinaster* Ait.). *New Forests* 39(3): 387-400.

Lichtenthaler, H.K. & Buschmann, C. 2001. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. *Current Protocols in Food Analytical Chemistry* 1(1): F4. 3.1-F4. 3.8.

Ling, A.P.K., Kok, K.M., Hussein, S. & Ong, S.L. 2009. Effects of plant growth regulators on adventitious roots induction from different explants of *Orthosiphon stamineus*. *Am-Eurasian Journal of Sustainable Agriculture* 3(3): 493-501.

Matile, P., Schellenberg, M. & Vicentini, F. 1997. Localization of chlorophyllase in the chloroplast envelope. *Planta* 201(1): 96-99.

Meir, S., Philosoph-Hadas, S., Gloter, P. & Aharoni, N. 1992. Nondestructive assessment of chlorophyll content in watercress leaves by a tristimulus reflectance colorimeter. *Postharvest Biology and Technology* 2(2): 117-124.

Morais, R.R.D., Gonçalves, J.F.D.C., Santos Júnior, U.M.D., Dünisch, O. & Santos, A.L.W.D. 2007. Chloroplastid pigment contents and chlorophyll a fluorescence in Amazonian tropical three species. *Revista árvore* 31: 959-966.

Mustroph, H., Stollenwerk, M. & Bressau, V. 2006. Current developments in optical data storage with organic dyes. *Angewandte Chemie International Edition* 45(13): 2016-2035. doi: 10.1002/anie.200502820

Nasarudin, N.A., Mohamad, J., Ismail, S. & Mispan, M.S. 2018. Effect of nitrogen, phosphorus and potassium (NPK) and bacterial bio-fertilizer on the antioxidant activity and chlorophyll content of aerobic rice. *Molecules* 23: 55.

Noseworthy, J. & Loy, B. 2008. Improving eating quality and carotenoid content of squash. In *Cucurbitaceae. Proceedings of the IXth EUCARPIA Meeting on Genetics and Breeding of Cucurbitaceae*, Avignon, France, 21-24 May 2008 (pp. 521-528). Institut National de la Recherche Agronomique (INRA).

Purbajanti, Endang Dwi, Widyati Slamet & Eny Fuskhah. 2019. Effects of organic and inorganic fertilizers on growth, activity of nitrate reductase and chlorophyll contents of peanuts (*Arachis hypogaea* L.). *IOP Conference Series: Earth And Environmental Science.* 250(1): 012048.

Ramasamy, P.K. & Suresh, S.N. 2010. Effect of vermicompost on root numbers and length of sunflower plant (*Helianthus annuus* L.). *Journal of Pure and Applied Microbiology* 4(1): 297-302.

Raya, K.B., Ahmad, S.H., Farhana, S.F., Mohammad, M., Tajidin, N.E. & Parvez, A. 2015. Changes in phytochemical contents in different parts of *Clinacanthus nutans* (Burm. f.) lindau due to storage duration. *Bragantia* 74(4): 445-452.

Roura, S.I., Davidovich, L.A. & del Valle, C.E. 2000. Postharvest changes in fresh Swiss chard (*Beta vulgaris*, type cycla) under different storage conditions. *Journal of Food Quality* 23(2): 137-147.

Sardoei, A.S., Roien, A., Sadeghi, T., Shahadadi, F. & Mokhtari, T.S. 2014. Effect of vermicompost on the growth and flowering of African Marigold (*Tagetes erecta*). *American-Eurasian Journal of Agriculture and Environmental Science*. 10.5829/idosi.aejaes.2014.14.07.12366

Sundararasu, K. & Neelanarayanan, P. 2012. Effect of vermicompost and inorganic fertilizer on the growth and yield of tomato, *Lycorpersiumesculentum* L. *International Journal of Current Research* 4(7): 49-51.

Uawonggul, N., Chaveerach, A., Thammasirirak, S., Arkaravichien, T., Chuachan, C., & Daduang, S. 2006. Screening of plants acting against *Heterometrus laoticus* scorpion venom activity on fibroblast cell lysis. *Journal of Ethnopharmacology* 103(2): 201-207.

Wolf, F.T. 1956. Changes in chlorophylls *a* and *b* in autumn leaves. *American Journal of Botany* 43(9): 714-718.

Wrolstad, R.E., Hong, V., Boyles, M.J. & Durst, R.W. 1995. Use of anthocyanin pigment analysis for detecting adulteration in fruit juices. *Methods to Detect Adulteration in Fruit Juice and Beverages* 1: 260-286.

Yang, H.S., Peng, T.W., Madhavan, P., Abdul Shukkoor, M.S. & Akowuah, G.A. 2013. Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf. *International Journal of Drug Development and Research* 5(3): 349-355.

Yusof, Z., Ramasamy, S., Mahmood, N. & Yaacob, J. 2018. Vermicompost supplementation improves the stability of bioactive anthocyanin and phenolic compounds in *Clinacanthus nutans* Lindau. *Molecules* 23(6): 1345.

Zainul Amiruddin Zakaria, Mohammad Hafiz Abdul Rahim, Norhafizah Mohtarrudin, Arifah Abdul Kadir, Manraj Singh Cheema, Zuraini Ahmad, Ching Siew Mooi, & Siti Farah Md Tohid. 2016. Acute and sub-chronic oral toxicity studies of methanol extract of *Clinacanthus nutans* in mice. *African Journal of Traditional, Complementary and Alternative Medicines* 13(2): 210-222.

Zucco, M.A., Walters, S.A., Chong, S-K., Klubek, B.P. & Masabni, J.G. 2015. Effect of soil type and vermicompost applications on tomato growth. *International Journal of Recycling of Organic Waste in Agriculture* 4(2): 135-141.

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